Transient expression of anti-VEGFR2 nanobody in *Nicotiana tabacum* and *N. benthamiana*

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Abstract

In human, the interaction between vascular endothelial growth factor (VEGF) and its receptor (VEGFR2) is critical for tumor angiogenesis. This is a vital process for cancer tumor growth and metastasis. Blocking VEGF/VEGFR2 conjugation by antibodies inhibits the neovascularization and tumor metastasis. This investigation designed to use a transient expression platform for production of recombinant anti-VEGFR2 nanobody in tobacco plants. At first, anti-VEGFR2-specific nanobody gene was cloned in a *Turnip mosaic virus* (TuMV)-based vector, and then, it was expressed in *Nicotiana benthamiana* and *Nicotiana tabacum* cv. Xanthi transiently. The expression of nanobody in tobacco plants were confirmed by reverse transcription-polymerase chain reaction (RT-PCR), dot blot, enzyme-linked immunosorbent assays (ELISA), and Western blot analysis. It was shown that tobacco plants could accumulate nanobody up to level 0.45% of total soluble protein (8.3 µg/100 mg of fresh leaf). This is the first report of the successful expression of the camelied anti-VEGFR2 nanobody gene in tobacco plants using a plant viral vector. This system provides a fast solution for production of pharmaceutical and commercial proteins such as anti-cancer nanobodies in tobacco plants.

Keywords Molecular farming · Cancer · Nanobody · Transient expression · Recombinant protein · *Nicotiana tabacum*

Abbreviations

TuMV	Turnip mosaic virus
VEGFR2	Vascular endothelial growth factor receptor
ELISA	Enzyme-linked immunosorbent assays
RT-PCR	Reverse transcription-polymerase chain reaction

Introduction

The production of proteins, biological macromolecules or cellular components, vaccines, drugs, and recombinant proteins by plants is called molecular farming. Plant production platforms (stable and transient expression systems) are cheap, safe, capable for fold and assemble complex proteins and post-translationally modifications, easily scalable and free from human pathogens and diseases when compared to common commercial expression systems, such as *Escherichia coli*, *Pichia pastoris*, *Saccharomyces cerevisiae* and mammalian cell lines (Fischer et al. 2004, 1999; Twyman et al. 2003). Tobacco (*Nicotiana tabacum*) is one of the best species for molecular farming. Since many of the different plant viruses can efficaciously infect *Nicotiana benthamiana*, it most widely used for produce recombinant proteins by viral vectors (Goodin et al. 2008).

Stable transformation is the common strategy for expression of foreign proteins in plants, but it has some problems such as escape of recombinant genes into nature and need a long time for transgenic plants generation. To overcome these problems, several alternative methods suggested including transient expression systems by plant viral

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expression vectors (Hefferon 2009; Scholthof et al. 1996). The genus *Potyvirus* (including the Turnip mosaic virus (TuMV)) as a positive-sense ssRNA viral vectors (with about 10 kb genome long) can infect widespread plant species. These types of viruses have autonomous replication and high levels of expression; therefore, they are considered for heterologous genes expression in plants (Chen et al. 2007).

Nanobodies are small molecules (15 kDa) with high affinity to bind with antigens, high solubility and stability and low immunogenicity, which these advantages make them beneficial for immunoassays and therapeutic applications (Buelens et al. 2010; Saerens et al. 2008). Cancer is one of the most important death reasons all over the world (Ismaili et al. 2007; Siegel et al. 2014). Cancer tumors need angiogenesis for growth, invasion, and metastasis. Therefore, blocking of angiogenesis is one of the most interesting strategies for overcoming cancer (Folkman 2007). Vascular endothelial growth factor receptor-2 (VEGFR2) is one of the tumor-associated receptors on the endothelial cells. VEGFR2 has tyrosine kinase activity, and its connection with Vascular Endothelial Growth Factor (VEGF) begins the process of cell proliferation, tube formation, tumor progression, and inhibition of apoptosis (Olsson et al. 2006; Plate and Risau 1995). Therefore, prevention of VEGF–VEGFR2 connection can inhibit neovascularization and tumor metastasis (Behdani et al. 2013).

In this research, *anti-VEGFR2* nanobody gene was introduced into the TuMV vector by replacing the *GFP* (jellyfish green fluorescent protein) gene. Tobacco plants inoculated with the recombinants TuMV and the nanobody was expressed. This system provides a fast and economic method for producing of pharmaceutical and commercial proteins in tobacco plants (*N. tabacum* and *N. benthamiana*).

Materials and methods

Plant material and growth conditions

Nicotiana benthamiana and *N. tabacum* cv. Xanthi plants were used as the host plants. Seeds were grown in pots containing autoclaved soil, including 30% perlite + 30% peat moss + 40% farm soil and they were kept at 25 °C in a phytotron under a 16/8 h light/dark cycle.

Construction of recombinant TuMV-Anti VEGFR2 vector

p35STuMVGFP construct kindly provided by Dr. Shyi-Dong Yeh, Plant Pathology Department, National Cheng Hsing University, Taichung, Taiwan having CaMV 35S promoter was used in this study. Camelied anti-VEGFR2 Nanobody (*3VGR19*) has been isolated and cloned by Behdani

et al. (2012). The *3VGR19* (Genebank accession number MF280918) fused with a 6X His tag at the C-terminal region was inserted into the TuMV vector. To subclone *3VGR19* gene into the TuMV viral vector, the VHH coding region was amplified using the forward (5'-ACG TCC ATG GTT CAA GTA CAA TTA CAA GAA TC-3') and reverse (5'-ATC GGC TAG CAG AAG ATA CAG TTA CTT G-3') primers containing *NcoI* and *NheI* restriction enzymes, respectively. Polymerase chain reaction (PCR) product was separated in a gel electrophoresis of 1.5% agarose, stained with SafeStain (Thermo Fisher Scientific, US), and extracted by a gel extraction kit (Qiagen, South Korea). Then, 300 ng of purified PCR product was digested with one unit each of *NcoI* and *NheI* (Thermo Fisher Scientific, US). Digested DNA fragments were sub-cloned into the double-digested TuMV vector for replacing the *GFP* gene with *3VGR19*. Resulted recombinant viral vector, pTuMV-3VGR19, was transferred into *E. coli* DH5 α competent cells (Sambrook and Russell 2001). Grown colonies were screened by PCR colony using the specific forward and reverse primers. Finally pTuMV-3VGR19 construct was analyzed by digesting with restriction enzymes and sequencing.

Tobacco plant inoculation

To express the VHH in tobacco plants, pTuMV-3VGR19 was mechanically inoculated on upper surface of two top leaves, using a cotton stick (10 μ g in 10 μ l per leaf) according to Hosseini et al. (2013) method. To express the VHH in tobacco plants, 10 μ l (1 μ g/ μ l) of pTuMV-3VGR19 and wild-type TuMV (as control) were mechanically inoculated on 2–3 fully expanded tobacco leaves using a cotton stick according to Hosseini et al. (2013) recommended. Plant samples harvested at 14 days post inoculation based on initially studies (data not shown) and Gleba et al. (2005).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from *N. benthamiana* and *N. tabacum* cv. Xanthi leaf tissues were extracted from inoculated and control (non-inoculated) plants using the Qiagen kit (South Korea) according to the manufacturer's instruction. Extracted RNA was treated with DNaseI and first-strand cDNA was synthesized using the RevertAid Reverse Transcriptase (Thermo Scientific, US). The first-strand cDNA was used as a template in the following PCR procedures: forward (5'-TCT AGA GAT GAT GAT GAT AAA CAAG-3') and reverse (5'-ATC GGC TAG CAG AAG ATA CAG TTA CTT G-3'). PCR cycling conditions were as follows: 94 °C for 3 min for initial denaturation; 35 cycles of 94 °C for 30S, 60 °C for 45S, and 72 °C for 30S; and 72 °C for 10 min for a final

extension. The amplified PCR products were analyzed by 1% TAE Agarose gel.

Protein extraction and SDS-PAGE

About 0.5 g tissues of inoculated tobacco leaves were harvested and frozen by liquid nitrogen. Then it was ground and powdered and subsequently extraction buffer [0.2M Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA), 100 mM sucrose, and 0.1 mM 2-mercapthoethanol] was added (Abdoli-Nasab et al. 2013). Samples were centrifuged at 12,000g in a 2 ml microcentrifuge for 20 min at 4 °C, and then supernatant was transferred to tubes. In addition, protein concentration determined by Bradford's assay (Bradford 1976). Proteins were denatured by boiling for 5 min and were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue (Laemmli 1970).

Detection of nanobody by immunoassays

Dot blot (Stott 1989), indirect enzyme-linked immunosorbent assay (ELISA) (Wang and Gonsalves 1990) and western blot assay (Gooderham 1984) were carried out to quantitative detection of the VHH protein in the inoculated tobacco plants at 14 days post inoculation. For Dot blot, 20 µg of sample proteins was transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia, Piscataway, NJ, USA), after drying, membrane incubated in blocking solution [bovine serum albumin (BSA) 1%] with shaking (30 rpm) for 1 h. Then the membrane was washed with the PBST (phosphate buffered saline (PBS) solution with the detergent Tween® 20) for 3 × 10 min. Afterwards, membrane was incubated with primary antibody (polyclonal rabbit anti-camel IgG) dilution of 1:1000 in PBS for 1 h at room temperature and after washing with PBST (3 × 10 min) followed by HRP-conjugated anti-rabbit IgG (1 h at room temperature), washing with PBST (3 × 10 min) and treatment with DAB (3,3' diaminobenzidine tetrahydrochloride) solution as substrate. The reaction was stopped by washing in distilled water, when the spots were visible.

For indirect ELISA assay, 50 ng proteins of inoculated and control (un-inoculated) plants was diluted with bicarbonate buffer (35 mM NaHCO₃, 15 mM Na₂CO₃, pH 9.6) and loaded into a microtiter plate overnight at 4 °C. Plate was washed with PBST for 3 × 10 min and background was blocked with blocking buffer (1% (w/v) BSA in 0.01 M PBST) for 1 h at 37 °C, then plate was washed thrice with PBST for 10 min. Primary antibody [polyclonal rabbit anti-camel IgG (Behdani et al. 2012)] dilution of 1:1000 in PBS was added and incubated for 1 h at 37 °C. The wells were washed with PBST and incubated with the mouse HRP-conjugated anti-rabbit IgG diluted to 1:3000 blocking buffer

[1% (w/v) BSA in 0.01 M PBST] for 1 h at 37 °C. Plate washed with PBST thrice and incubated with substrate solution [1%DAB (3, 3' Diaminobenzidine tetrahydrochloride), 0.01% H₂O₂, 200 mM citrate buffer, pH 3.95) at room temperature for 20 min in the dark. The reaction was stopped by addition of 3 N NaOH and plates were read at 450 nm using a micro plate reader (BioTek, USA). Different levels (0, 1, 3, 6, 12, 24, 48 and 96 ng) of purified Nanobody were used to drawing standard curve and estimate Nanobody expression levels in transformed plants. The statistical analysis was done by SPSS v22 for Windows (SPSS Inc., Chicago, USA).

For western blot, leaf extracted proteins (100 µg) boiled for 5 min and loaded on a His-Select column (Sigma), then they were separated by 12% SDS-polyacrylamide gel and transferred onto the nitrocellulose membrane by semi-dry electroblotting at 13 V for 1 h in a Bio-Rad semidry blotting system. Membrane was incubated in 25 ml of 5% (w/v) non-fat dry milk in PBST. Then membrane was washed with PBST for 3 × 10 min and incubated with primary antibody (polyclonal rabbit anti-camel IgG) at a 1:1000 dilution for 2 h. Membrane was washed thrice in TBST buffer (3 × 10 min) and followed by incubation in a 1:3000 dilution of secondary antibody (mouse HRP-conjugated anti-rabbit IgG) for 2 h. The membrane was washed 3 × 10 min in TBST and incubated with substrate solution [1% DAB (3, 3' Diaminobenzidine tetrahydrochloride), 0.01% H₂O₂, 200 mM citrate buffer, pH 3.95) at room temperature for 1 h in the dark.

Results

Vector construction

In this research, we have used complete TuMV virus as a viral vector with Cauliflower mosaic virus (CaMV) 35S promoter. The cauliflower mosaic virus 35S (CaMV35S) promoter has been used to enhance the strong expression of Nanobody (Fig. 1).

The camelied nanobody gene was amplified using PCR and after digestion with *Nco*I and *Nhe*I, inserted between corresponding sites into the TuMV vector. The correct insertion of camelied anti-VEGFR2 gene was verified by PCR (Fig. 2), digestion with restriction enzymes and sequencing.

RT-PCR

The pTuMV-Anti-VEGFR2 vector was introduced into *N. benthamiana* and *N. tabacum* cv. Xanthi leaves mechanically and 2 weeks after incubation, and tobacco plants leaves were harvested. Total RNA were extracted from inoculated and control (non-inoculated) plants, treated with DNase and the first-strand cDNA was synthesized. The presence of the nanobody gene in inoculated leaves was determined by

Fig. 1 Viral vector construct displaying the key features of TuMv-GFP used in this research to clone and express camelized anti-VEGFR2 nanobody. 35S promoter Cauliflower mosaic virus (CaMV) 35S promoter, 5' and 3' UTR 5' and 3' untranslated region, P1 RNA-silencing suppressor, Hc-Pro helper component proteinase protein, P3 protein 3, 6K1 6 kDa 1 protein, CIP cylindrical inclusion

protein, 6K2 6 kDa 2 protein, Nla-VPg viral genome-linked protein of nuclear inclusion protein a, Nla-Pro proteinase domain of Nia, Nib nuclear inclusion protein b, CP the virus coat protein gene. This vector double digested with NcoI and NheI restriction enzymes to remove the GFP gene from it

Fig. 2 Verifying insertion of camelized anti-VEGFR2 nanobody in TuMv vector. PCR amplification of anti-VEGFR2 nanobody; C-: negative control (TuMv-GFP), C+: positive control, Lanes 1–4: bacteria colonies, molecular weight marker (1 kb standard GeneRuler)

Fig. 3 RT-PCR amplification of a 341 bp fragment from the anti-VEGFR2 nanobody with specific primers. C-1: negative control (water template), C-2: negative control (RNA template), C-3: negative control [Wald type (non-transformed plant)], C+: positive control, lanes 1–2: *N. benthamiana* transformed plants, lanes 3–5: *N. tabacum* transformed plants, M: molecular weight marker (1 kb standard GeneRuler)

RT-PCR in inoculated and non-inoculated plants. Results indicated that the incubated plants have 341 bp expected RT-PCR bands. However, negative control (wild type) samples did not have similar bands (Fig. 3). This analysis confirms that TuMV can infect tobacco plants, amplify its genome, and could express its genes in experimental plants.

SDS-PAGE, dot blot, ELISA, and western blot

Total soluble protein was extracted from the transformed and untransformed (negative control) tobacco plants. SDS-PAGE analysis (data not shown) of the extracted proteins from transformed and non-transformed plants did not show any band with 15 kDa weight. The existence or absence of nanobody protein in transformed and non-transformed plant leaves was investigated with its specific antibody. Dot blot analysis (Fig. 4) indicated that expressed nanobody protein

Fig. 4 Dot blot analysis of camelized anti-VEGFR2 nanobody transient expression in *N. benthamiana* and *N. tabacum* cv. Xanthi leaves. C-: negative control [Wald type (non-transformed plant)], C+: purified bacterial anti-VEGFR2 nanobody protein, lanes 1–3: *N. benthamiana* transformed plants, lanes 4–6: *N. tabacum* transformed plants

recognized by specific antibody and developed brown color. The variance in color intensity in the transformed plants represents the difference in recombinant protein concentrations.

Furthermore, the expression of recombinant nanobody in transformed plants was confirmed by Western-blot analysis (Fig. 5). This method is an efficient method for confirmation the expression of the recombinant gene in plants. Results indicated that transformed plants expressed a 15 kDa protein according to the expected molecular mass of the camelid anti-VEGFR2 nanobody protein. However, the wild type plants did not express a 15 kDa protein.

ELISA assay used to determine the quantity of the expressed camelid anti-VEGFR2 nanobody. The linear standard curve was used to estimate the amount of the anti-VEGFR2 nanobody protein in the transformed lines. ELISA assay (Fig. 6) using an anti-camel antibody indicated that

Fig. 5 Western blotting analysis of anti-VEGFR2 nanobody protein transiently expressed in tobacco plants. C+: purified bacterial anti-VEGFR2 nanobody protein, C-: negative control [wild type (non-transformed plant)], lane 1: *N. benthamiana* transformed plants, lane 2: *N. tabacum* transformed plants, M: protein molecular weight marker

Fig. 6 Analysis of Camelid anti-VEGFR2 nanobody expression in tobacco plants by ELISA assay. C-: negative control [wild type (non-transformed plant)], 1–3: *N. benthamiana* transformed plants, lanes 4–9: *N. tabacum* transformed plants. Three independent examinations were performed to show the average and SE

the maximum accumulation of the camelid anti-VEGFR2 nanobody protein in transferred *N. benthamiana* and *N. tabacum* was 15.7 $\mu\text{g}/100\text{ mg}$ (approximately accounting for 0.45% of TSP) and 8.3 $\mu\text{g}/100\text{ mg}$ of leaves (approximately accounting for 0.2% of TSP), respectively.

Discussion

New blood vessel formation or angiogenesis plays a vital role in some physiological process such as embryogenesis and wound healing. On the other side, cancer metastasis and growth of cancer cells depend on angiogenesis (Borgström et al. 1998; Kazemi-Lomedasht et al. 2015; Takahashi et al. 1996); therefore, blocking neovascularization causes necrosis and inhibiting tumor growth and metastasis. VEGF is the main vascular growth factor, and it is an indicator for tumor growth and metastasis (Ferrara 2009). VEGFR1 and VEGFR2 are two tyrosine kinase receptors and interaction between them and VEGF is essential for tumor angiogenesis (Silva et al. 2011). VEGFR2 only express in vascular endothelial cells, so suppression of VEGF/VEGFR2 interaction inhibits new blood vessel formation (Behdani et al. 2012; Hong et al. 2004; Yancopoulos et al. 2000). Nanobodies (single variable domains in antibodies of some organisms such as camel) can act similar the normal antibody for detection or destroy tumors (Dumoulin et al. 2002; Rajabi-Memari et al. 2006; Revets et al. 2005). Previously, Behdani et al. (2012) have isolated and described camelid anti-VEGFR2 nanobody with high affinity and high specificity to target. In this research, we expressed the anti-VEGFR2 (3VGR19) nanobody via a viral transient expression vector in two tobacco species for the first time.

In this research, we used the pTuMV-Anti-VEGFR2 vector (Fig. 1). This vector encodes P1/HC-Pro, a viral silencing suppressor, which inhibits plant miRNAs functions

(He et al. 2008) and expresses considerable recombinant protein in various species such as *N. benthamiana*. In this study, anti-VEGFR2 gene was inserted between N1b (as an RNA-dependent RNA polymerase) and coat protein (CP). In addition, N1a protease recognition site prevailing naturally between N1b and CP. In general, open reading frame (ORF) of heterologous proteins inserting in the TuMV N-terminal (NT) region of CP (Ivanov et al. 2003), N-terminal region of HC-Pro (Beauchemin et al. 2005), or N-terminal region of P1 (Rajamaki et al. 2005), although no significant differences in expression level were observed between these two (HC-Pro and P1) insertion sites, and usually, NT CP insertion site has higher yield of the expressed recombinant protein in most of the host plants (Chen et al. 2007). In general, viral vectors are divided into two groups, complete viruses or deconstructed vectors. In the first case, fully functional viruses (wild type) are used as expression vector and selected genes of interest (GOI) fused to one of the virus genes such as coat protein (CP) or GOI is under the transcriptional control of wild-type virus strong promoter. These types of vectors can cause widespread infection and produce high-yield recombinant proteins. However, these vectors have some limitations such as GOIs size and inability to infect a large number of species. Second type of viral vectors only uses essential viral elements and they can infect widespread plants species and, in this method, need to have both the recombinant *Agrobacterium tumefaciens* and large-scale Agroinfection (Mortimer et al. 2015). Besides the protein size, high yield and cheap production of antibodies for diagnosis and immunotherapy applications are challenging. As a first report, Takamatsu et al. (1987) used Tobacco mosaic virus (TMV) as a vector for production of recombinant proteins in tobacco. Since that time, many new strategies have been introduced for expressing active antibodies in plants. Hiatt et al. (1989) expressed a functional antibody in tobacco leaves. In addition, nanobody against MUC1 mucin from *Camelus dromedarius* (Rajabi-Memari et al. 2006) and *Camelus bactrianus* (Ismaili et al. 2007) expressed in tobacco plant. Today, some plant viruses such as tobacco mosaic virus (TMV), potato virus X (PVX), cucumber mosaic virus (CMV), turnip mosaic virus (TuMV), and cowpea mosaic virus (CPMV) have been engineered to produce vaccines and therapeutic proteins (Yusibov et al. 2011). So far, the use of TuMV viral vectors for the expression of recombinant proteins in tobacco plants has not been reported, but some reporter genes such as GFP and GUS expressed in *Arabidopsis thaliana* (Tourinho et al. 2008) and *Brassica campestris* (Chen et al. 2007) have been expressed using this viral vector. Same average yield of expressed recombinant anti-VEGFR2 nanobody in tobacco plants in current research (8.3–15.7 µg/100 mg FW), in many studies such as Varsani et al. (2006), (0.3–10 µg/100 mg FW), Mechtcheriakova et al. (2006), (50–100 µg/100 mg FW);

(Voinnet et al. 2003), (0.5–34 µg/100 mg FW) recombinant proteins concentration are lower than economics thresholds for large-scale production of pharmaceutical proteins. To increase recombinant protein yield, some strategies such as inducible viral vectors (Mortimer et al. 2015), virus redesign and rebuild an integrated system (hybrid viral vectors) (Lico et al. 2008) and improved expression hosts (Gleba et al. 2007) can be used.

In this research, SDS-PAGE and staining with Coomassie brilliant blue could not detect recombinant protein. Dyballa and Metzger (2009) reported that SDS-PAGE method staining with Coomassie brilliant blue just permit to detection of approximately 10–30 ng and more protein concentration (depend on the type of protein). In this research, recombinant protein expression was less than this amount threshold; therefore, we could not detect it by SDS-PAGE.

ELISA assay (Fig. 6) indicated that the maximum accumulation of the recombinant protein in transferred *N. benthamiana* and *N. tabacum* was 0.45% and 0.2% of TSP, respectively. This protein accumulation was as much as that reported by Chen et al. (2007) and Matić et al. (2012). There is a strong correlation between *Nicotiana* species/varieties and the heterologous protein yield (Conley et al. 2011). It seems that inactivity of RNA-dependent RNA polymerase 6 (RDR6) protein in *N. benthamiana* permits the strongest virus replication in comparison the commercial cultivars such as *N. tabacum* cv. Xanthi (Yang et al. 2004). *N. benthamiana* biomass yield is one-third of commercial tobacco cultivars (such as Xanthi, Samson etc.), although its recombinant protein production is 2–10 times more than them; therefore, commercial tobacco cultivars cannot benefit economical choice for production of heterologous protein transiently (Nausch et al. 2012). In addition, transformation method is very important factor in transient expression systems, for example, *N. exigua*, an Australian tobacco species, expressed high level of the reporter gene when infected by *Agrobacterium* infiltration procedures, but while the viral-based module vector used, no GFP accumulation was detected (Sheludko et al. 2007).

Conclusion

In this work, we showed transient expression of camelized anti-VEGFR2 nanobody in two tobacco species (*N. benthamiana* and *N. tabacum* cv. Xanthi) leaves by TuMV-Anti-VEGFR2 vector. Results indicated that the recombinant TuMV-3VGR19 vector was able to synthesize a protein of the expected molecular weight (15 kDa). Moreover, recognition of the 3VGR19 nanobody by the rabbit polyclonal anti-camel in ELISA declared the 3VGR19 obtained the correct conformation. Tobacco plants can accumulate the nanobody up to level 0.45% of TSP. Between them, *N. benthamiana*

showed more recombinant protein accumulation level. This is the first report of the production of camelized anti-VEGFR2 nanobody in tobacco. The study promises new horizons for expressing and producing cheap and fast anticancer nanobodies using viral vectors based on TuMV in tobacco plants. Although for commercial purposes, expression system should be made to optimize.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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